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Lab resource: Stem Cell Line

Generation of a human iPSC line (IMEDEAi008-A) derived from natural homozygous CCR5-Δ32 PBMCs enriched in the pro-erythroblast population

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A B S T R A C T

A 32 base pair deletion in the C-C chemokine receptor type gene (CCR5-Δ32), the main Human Immunodeficiency Virus (HIV) co-receptor results in a non-functional protein. Individuals homozygous for the CCR5-Δ32 mutation are resistant to HIV infection. Here we report the generation, from pro-erythroblast enriched Peripheral Blood Mononuclear Cells (PBMCs) from a naturally occurring CCR5-Δ32/Δ32 individual, of the fully characterized iPSC line IMEDEAi008-A. The new line has normal karyotype, carry the Δ32 mutation in homozygosity, is free of plasmid integrations, express high levels of pluripotency markers and can differentiate into all three germ layers.

Resource Table

Unique stem cell line identifier	IMEDEAi008-A
Alternative name(s) of stem cell line	hiPS SCD 10.44 CL2
Institution	IMEDEA - Instituto Mediterráneo de Estudios Avanzados
Contact information of distributor	Daniel Bachiller; d.b@csic.es
Type of cell line	iPSCs
Origin	Human
Additional origin info	Age: N/A Sex: Female Ethnicity if known: Caucasian
Cell Source	Original cell type: Peripheral Blood Mononuclear Cell (PBMC)
Clonality	Clonal cell line
Method of reprogramming	Episomal Plasmids
Genetic Modification	NO
Type of Modification	N/A
Associated disease	N/A
Gene/locus	N/A
Method of modification	N/A
Name of transgene or resistance	N/A
Inducible/constitutive system	N/A
Date archived/stock date	18th June 2020

Resource Table (*continued*)

Cell line repository/bank	Registered in the Human Pluripotent Stem Cell Registry (https://hpscreg.eu).
Ethical approval	The donor blood sample was provided by the Biobank of Instituto de Investigaciones Sanitarias Illes Balears with the approval of the Comité Etico de Investigacion Clínica de las Ilslas Baleares (Spain). Approval number IB3422/17PI.

1. Resource utility

IMEDEAi008-A has been produced by reprogramming a pro-erythroblast enriched population obtained from PBMCs. Due to the differences in reprogramming technology and the fact that the epigenetic signature and gene expression pattern in progenitor blood cells like pro-erythroblast is closer to ESC than those of fibroblasts, IMEDEAi008A could be a good complement to the CCR5-Δ32/Δ32 fibroblast-derived iPSC lines recently described (Chen et al., 2019). In addition, its cellular origin could make it easier to use in hematopoietic differentiation than the fibroblast-derived iPSCs. This line represents a non-invasive, easy accessible and unlimited source of CCR5-Δ32/Δ32 iPSCs that could be differentiated to study the multiple roles of CCR5 in infectious diseases (Klein, 2008), or its potential use in cell therapy approaches for the treatment of AIDS.

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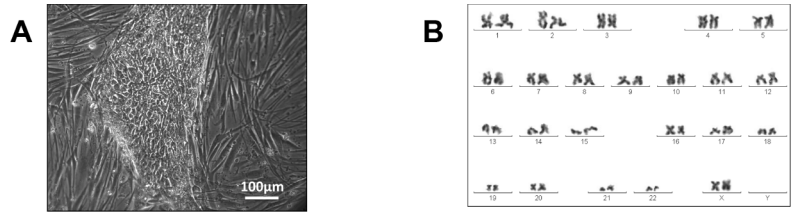

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Fig. 1. Characterization of a novel human iPSC line IMEDEAi008-A. (A) Phase contrast image of the iPSC line growing on passage 13 on a feeder-coated plate (scale bar = 100 μm). (B) Normal female karyotype of IMEDEAi008-A after G band analysis. (C) Sequencing of the CCR5 gene in primary PBMCs and IMEDEAi008-A to confirm the presence of the 32 bp deletion. (D) Immunofluorescence assay demonstrating the pluripotency phenotype of the line by the expression of SOX2, OCT3/4, TRA 1-60 and NANOG. (E) Flow cytometry analysis of SSEA4 and TRA 1-81 expression. (F) Immunofluorescence assay showing that after 10 days of directed differentiation IMEDEAi008-A cells express specific markers of ectoderm, mesoderm and endoderm. (G) The absence of episomal plasmid integration (EBNA) in IMEDEAi008-A was checked by PCR. GAPDH, housekeeping control for PCR analysis.

Table 1
Characterization and validation.

Classification	Test	Result	Data
Morphology	Photography	Normal appearance of packed iPS colonies. Scale bars: 100 μm	Fig. 1 panel A
Phenotype	Qualitative analysis by Immunocytochemistry	The cell line expressed the pluripotency markers: SOX2, OCT3/4, TRA-1-60 and NANOG.	Fig. 1 panel D
	Quantitative analysis by Flow Cytometry	The cell line expressed high levels of the pluripotency markers SSEA-4 and TRA-1-81	Fig. 1. Panel E
Genotype	Karyotype (G-banding) and resolution	IMEDEAi008-A: 46, XX Resolution 450–500	Fig. 1. Panel B
Identity	Microsatellite PCR analysis	Not performed	
	STR analysis	The STR profiles of cell line matched 100% with that of the parental PBMCs cells (10 loci analyzed)	Submitted in archive with journal
Mutation analysis (IF APPLICABLE)	Sequencing	Homozygous delta32 deletion of CCR5 (CCR5-Δ32/Δ32)	Fig. 1. Panel C
Microbiology and virology	Mycoplasma	Mycoplasma testing by PCR using MycoSPY Kit (Biontex).	Submitted in archive with journal
Differentiation potential	Directed differentiation	The cell line differentiated into the three germ layers including ectoderm (Nestin, Pax6 and TUJ-1), mesoderm (GATA-4, α-SMA and Vimentin) and endoderm (Sox17, AFP and FoxA2).	Fig. 1. Panel F
Donor screening (OPTIONAL)	NA	NA	NA
Genotype additional info (OPTIONAL)	NA	NA	NA

2. Resource details

To date, there is no cure for HIV infection. Anti-retroviral therapies can control viral replication but not eradicate the virus, which results, at best, in the chronification of the disease. To solve the situation, several cell and gene-based therapies have been tried. Genetic modification of autologous HSCs and T-cells is laborious, time-consuming and entails the risks commonly associated to genetic engineering. On the other hand, although the efficiency of allogeneic CCR5-Δ32/Δ32 HSCs transplantation has been demonstrated, the scarcity of homozygous donors constitutes an important limitation for its wide application.

One possible way out of the problem could be the use of iPSCs derived from naturally occurring CCR5-Δ32/Δ32 donors as an unlimited source of material for the production of HSCs.

IMEDEAi008-A was obtained by OriP/EBNA (Epstein-Barr nuclear antigen-1) episomal plasmid reprogramming (Okita et al., 2011) (OCT3/4, SOX2, KLF4, L-MYC, LIN28, BCL-xL and shp53) of natural CCR5-Δ32/Δ32 pro-erythroblast enriched PBMC population. Pro-erythroblast ($CD36^+/CD71^+$) expansion (Supplementary Fig. S1) during 7 days of culture (Supplementary Fig. S2) before reprogramming increased several folds their number and at the same time reduced drastically the percentage of cells with V(D)J genetic rearrangements. The resulting iPSC line showed morphology (Fig. 1A) and growth behavior typical of human Embryonic Stem Cells (hESC) indicating that CCR5 is not required during reprogramming. The new line also displayed normal female karyotype (46, XX) (Fig. 1B). In addition, after PCR analysis using specific primers (Table 2) that amplify the common element in all plasmids: OriP/EBNA, it was confirmed the absence of plasmid integration (Fig. 1G). Short tandem repeat (STR) analysis (Table 1, data not shown) indicated a 100% match with parental PBMCs. DNA sequencing was used to identify the delta 32 (Δ32) deletion in CCR5 (Fig. 1C). Regarding stemness, immunocytochemical analysis demonstrated the expression of the pluripotency-associated markers: SOX2, OCT3/4, TRA 1-60 and NANOG (Fig. 1D), while SSEA-4 and TRA-1-81 expression was confirmed by quantitative flow cytometry (Fig. 1E). Finally, directed differentiation into all three germ layers proved the differentiation potential of the line. The expression of specific markers for ectoderm, mesoderm and endoderm in the differentiated cells was analyzed by immunocytochemistry (Fig. 1F). Absence of mycoplasma was also assessed (Supplementary Fig. S3).

In summary, IMEDEAi008-A is a very interesting CCR5-Δ32 homozygous iPSC line with possible applications in the study of HIV infection pathophysiology as well as in the development of HSC-based allogeneic cell therapies.

3. Materials and methods

3.1. iPSCs derivation and expansion

Peripheral Blood Mononuclear Cells (PBMCs) were isolated by ficoll from a natural homozygous CCR5-Δ32 donor peripheral blood sample. PBMCs were cultured in a pro-erythroblast serum-free specific media (1:1 IMDM/Ham's F12 supplemented with: 1% ITS-X, 1% Chemically Defined Lipid Concentrate, 1% Glutamax, 0.025 g/L L-Ascorbic Acid, 5 g/L BSA, 100 ng/ml SCF, 10 ng/ml IL3, 2U/ml EPO, 40 ng/ml IGF-1, 1 μM Dexamethasone and 100 μg/ml Holo-transferrin). After 7 days of culture, 8×10^6 cells were nucleofected with four OriP/EBNA1-based episomal plasmids: 2 μg of pCXLE-hOCT3/4-shp53, 2 μg of pCXLE-hSK and 2 μg of pCXLE-hUL (Vallejo-Diez et al., 2019) in addition to 2 μg of GBX. Cells were cultured on one p12 well and pro-erythroblast complete medium supplemented with 10 μM Rock Inhibitor Y-27632 for 48 h. Reprogrammed cells were counted and 5×10^5 cells/well were seeded on p6 well plates coated with MEF-CD1 feeders. Culturing media was gradually changed from pro-erythroblast to hiPS medium: Knockout DMEM (Invitrogen) supplemented with 20% Knockout Serum Replacement (Invitrogen), 2 mM GlutaMAX, 1% NEAA, 50U/ml Penicillin, 50 mg/ml Streptomycin, 0,1m Mercaptoethanol and 8 ng/ml bFGF.and. From day 6 after reprogramming, NaB (0.5 mM) and Vitamin C (50 μg/ml) were added to the media.

After 18 days in culture, iPS colonies were manually isolated and grown on MEF CD1 until passage 3 when cells where seeded on human

Table 2
Reagent details.

Antibodies used for immunocytochemistry			
	Antibody	**Dilution**	**Company Cat # and RRID**
Pluripotency Markers	Mouse anti-OCT4	1:50	Santa Cruz Biotech; sc-5279; RRID:AB_628051
	Rabbit anti-NANOG	1:200	Cell Signaling; D73G4; RRID:AB_10559205
	Mouse anti-SOX2	1:100	R and D Systems; MAB2018;
	Mouse anti-TRA-1-60	1:100	RRID:AB_177629 Millipore; MAB4360; RRID:AB_2119183
Differentiation Markers	Rabbit anti-Nestin	1:500	Sigma-Aldrich; N5413; RRID:AB_1841032
	Rabbit anti-TUJ-1	1:500	Covance; MRB-435P-100; RRID:AB_663339
	Mouse anti-Pax6	1:100	DSHB pax6, RRID:AB_528427
	Mouse anti-GATA-4	1:300	Santa Cruz Biotech; sc-25310; RRID:AB_627667
	Mouse anti-Vimentin	1:100	Abcam; ab80667 RRID:AB_1603290
	Mouse anti-α-SMA	1:200	Sigma-Aldrich; A2547 RRID:AB_476701
	Goat anti-SOX-17	1:100	R&D Systems; AF1924; RRID:AB_355060
	Rabbit anti-AFP	1:200	Dako; A0008; RRID:AB_2650473
	Rabbit anti-FoxA2	1:400	Cell Signaling; 8186 RRID:AB_10891055
Secondary antibodies	Alexa Fluor 555 Donkey Anti-Rabbit IgG	1:500	Invitrogen; A-31572; RRID:AB_162543
	Alexa Fluor 555 Donkey Anti-Mouse IgG	1:500	Invitrogen; A-315700 RRID:AB_2536180
	Alexa Fluor 488 Donkey Anti-Mouse IgG	1:500	Invitrogen; A-21202; RRID:AB_141607
	Alexa Fluor 555 Donkey Anti-Goat IgG (tiene q sere n 555)	1:500	Invitrogen; A-21432, RRID: AB_2535853
	Alexa Fluor 488 Donkey Anti-Rabbit IgG	1:500	Invitrogen; A-21206 RRID:AB_141708
Antibodies used for Flow Cytometry			
	Antibody	**Vol. per test**	**Company Cat # and RRID**
Pluripotency Markers	Mouse Anti TRA-1-81, APC	20 µl	BD Biosciences; 560793; RRID:AB_10550550
	Mouse Anti SSEA-4, PE	20µl	BD Biosciences; 560128 RRID:AB_1645533
	Mouse IgM, k Isotype Control, APC	1 µl	BD Biosciences; 560806; RRID:AB_2034030
	Mouse IgG3, k Isotype Control PE	1 µl	BD Biosciences; 559926 RRID:AB_10050453
Primers			
	Target	**Forward/Reverse primer (5′-3′)**	
Episomal Plasmids Integration PCR	EBNA	ATCGTCAAAGCTGCACACAG/CCCAGGAGTCCCAGTAGTCA	
House-Keeping Genes (PCR)	GAPDH	GCACCGTCAAGGCTGAGAAC/AGGGATCTCGCTCCTGGAA	
Sequencing	CCR5	TTAAAAGCCAGGACGGTCAC/TGTAGGGAGCCCAGAAGAGA	

HFF1-W3R feeders. Rock Inhibitor Y-27632 was used at 10 µM during the passages. Cells were cultured at 37 °C and 5% CO_2 in a humidified incubator.

3.2. Karyotype analysis

G-banded metaphases analysis was carried out by *Biobanco del Sistema Sanitario Público de Andalucía* (Granada, Spain).

3.3. Reprogramming plasmid integration analysis

PCR analysis was used to detect a common element of all the reprogramming plasmids: OriP/EBNA-1. Amplification reactions were carried according to standard protocols with WonderTaq (Euroclone).

3.4. STR analysis and sequencing

After DNA isolation, CCR5 was amplified by PCR using EuroTaq (Euroclone) and primers detailed in Table 2. Amplicons were sequenced by SECUGEN S.L (Madrid) using a 96-capillary DNA analyser Abi 3730. STR analysis was performed by the Genomics Core facility at the Instituto de Investigaciones Biomédicas (IIBM, Madrid).

3.5. In vitro directed differentiation

Ectoderm, mesoderm and endoderm differentiation was performed as described previously (Vallejo-Diez et al., 2019). After 10 days of culture cells were fixed in PFA 4% for immunocytochemistry.

3.6. Immunofluorescence staining

Undifferentiated iPSCs (for pluripotency markers assays) and differentiated cells (for differentiation potential assays) were washed with PBS, fixed for 20 min, washed again with PBS and permeabilized with 0,2% Triton X-100 and 100 mM glycine in PBS for 30 min at room temperature (RT). To block non-specific binding sites, PBS 5% BSA was added an incubated for 60 min at RT. Primary antibodies incubation was done overnight at 4 °C in PBS 2% BSA. After three washes with PBS, cells were incubated with fluorescence-conjugated secondary antibodies in PBS 2% BSA for 60 min at RT and darkness. The antibodies used are described in Table 2. After washing with PBS, cells were stained with DAPI for 5 min at RT in darkness.

3.7. Flow cytometry analysis of pluripotency marker

Cells were detached with Tryple and incubated 45 min and 4 °C with conjugated antibodies (Table 2) against cells surface markers TRA-1-81 and SSEA-4. The corresponding isotype antibodies were used as controls. Influx Cell Sorter and BD 1.0.0.650 Software were used for the cytometry analysis.

3.8. Mycoplasma detection

Mycoplasma testing was carried out using the MycoSPY Mycoplasma PCR detection kit (Biontex), which detects a wide range of bacteria from the *Mollicutes* class. An Internal Control template was included to discard the presence of PCR inhibitors and rule out false positive results.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scr.2020.101918.

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